

# Cleavage of interleukin 1 $\beta$ (IL-1 $\beta$ ) precursor to produce active IL-1 $\beta$ by a conserved extracellular cysteine protease from *Streptococcus pyogenes*

(pyrogenic exotoxin B/toxin/toxic-shock-like syndrome/cytokine)

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**ABSTRACT** Streptococcal pyrogenic exotoxin B (SPE B), a conserved extracellular cysteine protease expressed by the human pathogenic bacterium *Streptococcus pyogenes*, was purified and shown to cleave inactive human interleukin 1 $\beta$  precursor (pIL-1 $\beta$ ) to produce biologically active IL-1 $\beta$ . SPE B cleaves pIL-1 $\beta$  one residue amino-terminal to the site where a recently characterized endogenous human cysteine protease acts. IL-1 $\beta$  resulting from cleavage of pIL-1 $\beta$  by SPE B induced nitric oxide synthase activity in vascular smooth muscle cells and killed cells of the human melanoma A375 line. Two additional naturally occurring SPE B variants cleaved pIL-1 $\beta$  in a similar fashion. By demonstrating that SPE B catalyzes the formation of biologically active IL-1 $\beta$  from inactive pIL-1 $\beta$ , our data add a further dimension to an emerging theme in microbial pathogenesis that bacterial and viral virulence factors act directly on host cytokine pathways. The data also contribute to an enlarging literature demonstrating that microbial extracellular cysteine proteases are important in host-parasite interactions.

*Streptococcus pyogenes* strains express several extracellular proteins that are involved in virulence. Among these molecules are three pyrogenic exotoxins, designated A, B, and C (1). Streptococcal pyrogenic exotoxins (SPEs) share many biological properties, including fever induction and the ability to enhance susceptibility to endotoxic shock (1). SPE A and SPE C are bacteriophage-encoded superantigens and their structural genes (*speA* and *speC*) are variably present in natural isolates (2, 3). In contrast, the SPE B structural gene (*speB*) is found in all isolates of the species and is presumed to be chromosomally encoded (4). SPE B is a cysteine protease that is released extracellularly as a zymogen of about 40 kDa (6, 7). The mature protease ( $\approx$ 27.6 kDa) is formed by autoproteolytic truncation of the zymogen.

Significant evidence has accumulated over several decades that SPE B is an important streptococcal virulence factor. (i) In the initial characterization of the molecule, Elliott (6) showed that the enzyme has fibrinolytic activity. (ii) Björck *et al.* (8) found that a cysteine protease inhibitor suppressed bacterial growth *in vitro* and protected mice from lethal intraperitoneal streptococcal inoculation. (iii) Purified SPE B injected into rabbits causes myocardial necrosis (9). (iv) Patients with fatal streptococcal infection have lower acute antibody levels to SPE B than do individuals with less severe infections (10). (v) Sequencing of the SPE B structural gene from 67 *S. pyogenes* strains selected to represent organisms with divergent chromosomal genotypes, as assessed by multilocus enzyme electrophoresis and M protein serotypes, identified only nominal allelic variation (V.K. and J.M.M.,

unpublished data), a result that implies evolutionary pressure has constrained diversity at the *speB* locus.

Interleukin 1 $\beta$  (IL-1 $\beta$ ), a major mediator of inflammation, is synthesized as an inactive precursor by monocytes, endothelial cells, and other tissues (11). Recently, a human cysteine protease (termed ICE) was described (12, 13) that converts the 31-kDa inactive IL-1 $\beta$  precursor (pIL-1 $\beta$ ) to the 17.5-kDa mature active IL-1 $\beta$  (mIL-1 $\beta$ ) by cleaving between Asp-116 and Ala-117 (14, 15). Monocytes also release pIL-1 $\beta$  extracellularly in response to several activating signals and cell injury leading to necrosis (16, 17). Inasmuch as several of the signs and symptoms observed in *S. pyogenes* infections may be due to increased cytokine activation (18), we tested the hypothesis that SPE B functionally mimics ICE and converts pIL-1 $\beta$  to biologically active mIL-1 $\beta$ .

## MATERIALS AND METHODS

**Bacterial Strains and Cysteine Protease Variants.** Strain MGAS 1719 is identical to strain 5797 described by Elliott and co-workers (6, 19, 20). This organism is also known as Lancefield strain B220 (21) and was obtained from K. H. Johnston (Louisiana State University, New Orleans). Strain MGAS 1719 has the *speB7* allelic variant of the cysteine protease gene (V.K. and J.M.M., unpublished data). Toxin purified from this organism was used in most of the experiments. Streptococcal protease was also purified from strains MGAS 279 and MGAS 289, which synthesize the *speB2* and *speB11* variants, respectively. The predicted amino acid sequence of SPE B made by MGAS 289 differs from that made by MGAS 1719 at residues 111 (Ala  $\leftrightarrow$  Val), 308 (Ser  $\leftrightarrow$  Gly), and 317 (Ser  $\leftrightarrow$  Ala), and there is one predicted amino acid difference (Ala  $\leftrightarrow$  Val at residue 111) between SPE B made by MGAS 279 and MGAS 1719.

**Purification of the Cysteine Protease.** Bacteria were grown overnight at 37°C in 5% CO<sub>2</sub> on brain-heart infusion (BHI) agar. The overnight culture was used to inoculate 200 ml of BHI liquid medium, and the culture was incubated for 12–14 hr at 37°C in 5% CO<sub>2</sub>. A 50-ml aliquot of the overnight growth was added to 2 liters of chemically defined medium (JRH Biosciences, Lenexa, KS), pH 6.0, and the culture was incubated at 37°C in 5% CO<sub>2</sub>. The broth was maintained at pH 5.5–6.0 by the addition of sterile sodium bicarbonate (10%, wt/vol). After 8–9 hr the cells were removed by centrifugation and the supernatant was concentrated to 250 ml by passage through a 10-kDa cutoff spiral ultrafiltration cartridge (Amicon). Buffer exchange (>99%) by diafiltration was conducted with 1.5 liters of 20% ethanol/20 mM

Abbreviations: SPE, streptococcal pyrogenic exotoxin; IL-1 $\beta$ , interleukin 1 $\beta$ ; pIL-1 $\beta$ , precursor IL-1 $\beta$ ; mIL-1 $\beta$ , mature IL-1 $\beta$ ; NOS, nitric oxide synthase; SMC, smooth muscle cell; TSLs, toxic-shock-like syndrome.

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Tris-HCl, pH 7.0 (buffer A), at 4°C, and the material was stored overnight at 4°C. The diafiltered solution was passed through a matrex gel red A (Amicon) column (1.5 cm × 15 cm) equilibrated with buffer A. The column was washed with buffer A until the absorption (280 nm) returned to baseline, and the protein was eluted with buffer A containing 2 M NaCl. The eluted material was collected as one fraction and concentrated to 3 ml by ultrafiltration (Centriprep 10, Amicon), and the buffer was exchanged with phosphate-buffered saline (PBS, pH 7.2) by gel-filtration chromatography (Bio-Rad).

**Assay for pIL-1β Cleavage.** Two distinct assays were used to test the ability of streptococcal cysteine protease to cleave human pIL-1β. One assay employed radiolabeled pIL-1β (generously provided by S. M. Molineaux and M. J. Tocci, Merck Research Laboratories) made in a rabbit reticulocyte transcription-translation system as substrate (13). Briefly, a 0.5-μl aliquot of [<sup>35</sup>S]methionine-labeled pIL-1β was incubated with 0.25 μg of purified protease in a total volume of 20 μl of buffer (100 mM Hepes, pH 7.0/10% sucrose/0.1% Chaps/5 mM dithiothreitol) at 30°C for 1 hr. A 10-μl aliquot of the mixture was resolved by SDS/PAGE and the results were analyzed by autoradiography.

In the second assay, purified human recombinant pIL-1β (100 ng) (22) was incubated with 5–500 ng of purified streptococcal protease at 37°C for 30 min in PBS (pH 7.4), and the mixture was resolved by SDS/PAGE and transferred to nitrocellulose (Hybond ECL, Amersham). The membrane was blocked with 5% dried milk dissolved in PBS/Tween 20 for 1 hr and incubated for 1 hr with a 20 μg/ml solution of a monoclonal antibody (16F5) specific for the carboxyl terminus of IL-1β (22). After incubation with a 1:5000 dilution of sheep anti-mouse horseradish peroxidase-conjugated antibody (Amersham), the blot was visualized with enhanced chemiluminescence developing materials according to the manufacturer's instructions. The results were analyzed by autoradiography, with a 10-sec exposure.

**Assay for Nitric Oxide Synthase (NOS) Activity.** Rat aortic smooth muscle cells (SMCs) were grown to confluence in 24-well trays (Costar) in Waymouth's MB/752-1 medium supplemented with 5% fetal bovine serum. Cell layers were washed three times with serum-free Waymouth's medium (SFM) and then incubated in SFM for 24 hr in the presence or absence of SPE B, mIL-1β, pIL-1β, or pIL-1β with SPE B. After 24 hr, conditioned media were collected, centrifuged briefly to remove detached cells and debris, and assayed directly for nitrite anion levels (23). Nitrite concentrations of conditioned medium samples were determined by comparison to a standard curve generated with sodium nitrite. In some cases cells were incubated with authentic IL-1β (R & D Systems) at a concentration previously shown to maximally induce NOS activity over 24 hr by rat aortic SMCs (24).

**A375 Cell Line Assay for IL-1β.** A375 is a human melanoma cell line against which IL-1β exerts a cytotoxic effect (25). SPE B was incubated with recombinant purified pIL-1β for 30 min at 37°C. The reaction was terminated by the addition of ice acetate, and the mixture was desalted by gel-filtration chromatography. Uncleaved pIL-1β, SPE B alone, and mIL-1β were used as controls.

**Protein Sequencing.** Amino-terminal protein sequencing was performed with an Applied Biosystems model 477A instrument (Protein Sequencing Facility, Baylor College of Medicine, Houston, TX).

## RESULTS

**Purification of SPE B.** SDS/PAGE and Coomassie blue staining of the proteolytically active material eluted from the red dye A column showed a single major band with an apparent molecular mass of ≈30 kDa (Fig. 1). Reverse-phase

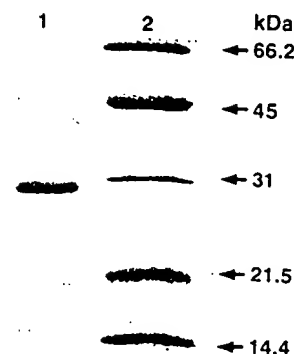


FIG. 1. Purified streptococcal cysteine protease. Streptococcal cysteine protease was purified from strain MGAS 1719 and resolved by SDS/PAGE. Lane 1, 2 μg of the purified protease; lane 2, molecular mass standards (Bio-Rad).

high-performance liquid chromatographic analysis of the purified protease with a C<sub>8</sub> analytical column also showed a single major protein peak corresponding to the ≈30-kDa molecule. The purified protein derived from dye-ligand affinity chromatography was blotted to Problott (Applied Biosystems) according to the manufacturer's instructions, stained with Coomassie blue, excised from the gel, and applied to an Applied Biosystems model 477A protein sequencer (Protein Sequencing Facility, Baylor College of Medicine). The sequence of the first 10 amino-terminal residues (QPVVKSLLDS) confirmed the identity of the purified material as streptococcal cysteine protease (26).

**Cleavage of pIL-1β by Streptococcal Cysteine Protease.** To determine if SPE B cleaves pIL-1β, we first used an assay employing radiolabeled pIL-1β made in a rabbit reticulocyte transcription-translation system (13). SPE B produced a cleavage product of ≈18 kDa, a size very similar to the apparent molecular mass of mIL-1β (Fig. 2A). Western blot analysis of the cleavage products generated from recombinant pIL-1β made in *E. coli* confirmed this result (Fig. 2B).

We then tested SPE B for ability to cleave a human pIL-1β mutant (Asp-116 → Ala-116, to create an Ala-116-Ala-117 linkage) (13) that is not degraded by ICE. As observed with wild-type pIL-1β, SPE B cleaved the mutant substrate to form a product with an apparent molecular mass of ≈18 kDa (Fig. 2A). Together, the results suggested that the substrate specificity of SPE B is different from that of ICE and that cleavage of pIL-1β by SPE B does not require Asp-116, as needed for cleavage by ICE.

To determine exactly where SPE B cleaved pIL-1β, we sequenced the amino-terminal 10 amino acid residues of the ≈18-kDa product made by degradation of recombinant pIL-1β. The results showed that SPE B cleaved pIL-1β between His-115 and Asp-116 to create a molecule 1 amino acid residue longer than mIL-1β.

**Normal Biological Activity of the mIL-1β Cleavage Product.** Because a highly active form of mIL-1β with Asp-116 at the amino terminus was described in the course of characterization of a metalloprotease found in human peripheral blood mononuclear cells (27), the data suggested that SPE B was processing inactive pIL-1β to biologically active IL-1β. We tested this hypothesis directly with two approaches. mIL-1β is a potent inducer of NOS activity in vascular SMCs (24). We added SPE B in the presence or absence of pIL-1β to confluent cultures of SMCs and assayed NOS activity by measuring nitrite anion levels in the medium after 24 hr. Neither SPE B nor pIL-1β alone produced a significant increase in nitrite levels; in contrast, addition of SPE B and pIL-1β together caused an ≈60-fold increase in nitrite accumulation (Fig. 3).

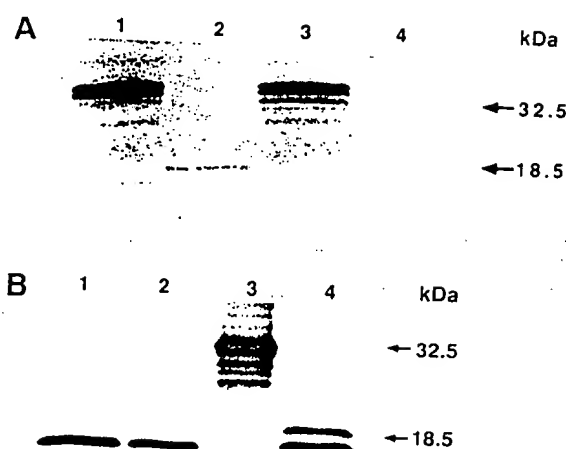


FIG. 2. Cleavage of human pIL-1 $\beta$  by streptococcal cysteine protease. (A) [ $^{35}$ S]Methionine-labeled pIL-1 $\beta$  (lane 1) was synthesized in a rabbit reticulocyte lysate system (13, 14) and incubated with 250 ng of purified SPE B (lane 2) or boiled SPE B (lane 3) for 1 hr. A human pIL-1 $\beta$  Asp-116  $\rightarrow$  Ala-116 mutant was also cleaved by SPE B (lane 4). The reduced intensity of the band corresponding to the mIL-1 $\beta$  molecule, relative to that of pIL-1 $\beta$ , is in part due to the occurrence of 12 methionine residues in pIL-1 $\beta$  versus only 6 methionine residues in mIL-1 $\beta$ . The difference in band intensity can also be attributed to nonspecific degradation of mIL-1 $\beta$  by either SPE B or proteases in the reticulocyte assay mixture. (B) Western immunoblot analysis of mature and recombinant pIL-1 $\beta$  cleavage products. Lane 1, mIL-1 $\beta$  alone; lane 2, mIL-1 $\beta$  plus SPE B; lane 3, pIL-1 $\beta$  alone; lane 4, pIL-1 $\beta$  plus SPE B. Incubations were conducted for 30 min at 37°C. The cleavage products were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with carboxyl-terminal-specific monoclonal antibody (22). The human recombinant pIL-1 $\beta$  substrate used in these assays was purified from a cell lysate of an *Escherichia coli* transformant containing the pIL-1 $\beta$  structural gene, by sequential column chromatography with Q-Sepharose, Procion Red-agarose, and phenyl-Sepharose CL-4B (22). The immunoreactive proteins greater and less than  $\approx$ 33 kDa in lane 3 are produced in the process used to make pIL-1 $\beta$  and are copurified with it. The lower band in lanes 2 and 4 has an apparent molecular mass of  $\approx$ 10 kDa. The  $\approx$ 18.5-kDa product is converted to the  $\approx$ 18-kDa molecule (not the  $\approx$ 10-kDa molecule) upon further incubation.

IL-1 $\beta$  generated by SPE B cleavage of pIL-1 $\beta$  was also found to be active in the A375 cell line assay (25). In an assay in which  $\approx$ 500 ng/ml of intact pIL-1 $\beta$  was inactive, a SPE B

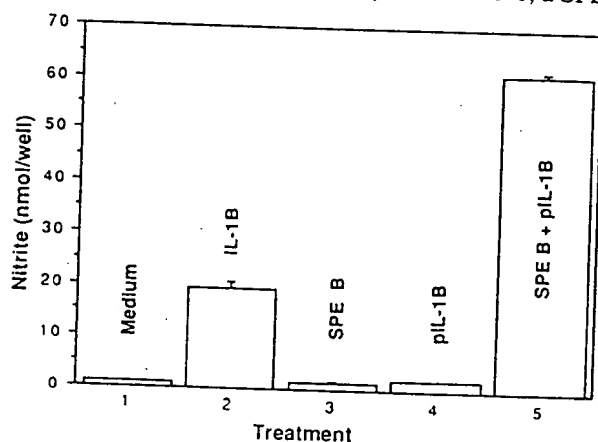


FIG. 3. Stimulation of NOS activity by SPE B and pIL-1 $\beta$  in rat aortic SMCs. Cells were treated for 24 hr with either SFM (1), mature IL-1 $\beta$  (3 ng/ml) (2), SPE B (4  $\mu$ g/ml) (3), pIL-1 $\beta$  ( $\approx$ 200 ng/ml) (4), or SPE B plus pIL-1 $\beta$  (5). The nitrite concentration in conditioned medium samples from treated cells was determined by comparison with a sodium nitrite standard curve.

digest of this material yielded  $6.1 \times 10^4$  units/ml of activity; 500 ng/ml of authentic IL-1 $\beta$  corresponded to  $1.1 \times 10^5$  units in this assay. Inasmuch as 500 ng of pIL-1 $\beta$  would yield about 275 ng of mIL-1 $\beta$ , these results suggest that SPE B catalyzes essentially quantitative conversion of pIL-1 $\beta$  to mIL-1 $\beta$ .

**Cleavage Activity of Variant SPE B Enzymes.** Two additional naturally occurring SPE B allelic variants (SPE B2 and SPE B11) also produced an IL-1 $\beta$  fragment with an apparent molecular mass identical to that made by SPE B7 purified from MGAS 1719 (Fig. 4).

## DISCUSSION

We have demonstrated that *S. pyogenes* cysteine protease converts inactive pIL-1 $\beta$  to biologically active IL-1 $\beta$ . Although our data do not directly address the *in vivo* significance of this discovery, we believe the finding may be particularly relevant to the pathogenesis of streptococcal infections, including toxic-shock-like syndrome (TSLs), for several reasons. Recent work has shown that strains recovered from most patients with TSLs express SPE A (14, 28). SPE A and streptolysin O (a membrane-damaging thiol-activated toxin) are potent inducers of tumor necrosis factor  $\alpha$  and IL-1 $\beta$  release and act synergistically to stimulate monocyte IL-1 $\beta$  production (29). In addition, human monocytes contain high levels of pIL-1 $\beta$  and release pIL-1 $\beta$  when stimulated (17). In principle, when pIL-1 $\beta$  is released extracellularly as a consequence of streptococcal stimulation, or cell necrosis, the bacterial cysteine protease could gain access to pIL-1 $\beta$  and increase mIL-1 $\beta$  levels.

Some *S. pyogenes* infections are characterized by several phenotypes that are consistent with significant increases in local and systemic mIL-1 $\beta$ . First, local inflammation occurs that is frequently accompanied by a brisk and intense pyrogenic response. Second, severe invasive infection with *S. pyogenes*, including TSLs (18), is characterized by hypotension, shock, multiorgan failure, hypocalcemia, and hypoalbuminemia, all of which are typical responses to increased systemic levels of IL-1 $\beta$  (11). In addition, especially severe disease would occur if there is host soft tissue destruction (such as recorded in many patients with TSLs), in part as a consequence of release of the large reservoir of pIL-1 $\beta$  from keratinocytes (30). We also note that streptococcal disease is unusually severe when it occurs concurrently with chickenpox and certain other viral exanthema characterized by epithelial destruction (31, 32).

Although our hypothesis provides a plausible explanation for the pathology recorded in some patients with TSLs, by itself, it does not adequately explain the recent increase in frequency of severe streptococcal invasive infections documented in the United States and several European countries (4, 10, 18). If, as we hypothesize, SPE B is involved in some cases of TSLs, either an increase in level of SPE B expression by streptococcal strains or a decrease in anti-SPE B antibody could contribute to temporal variation in disease frequency and severity. It therefore may be relevant that

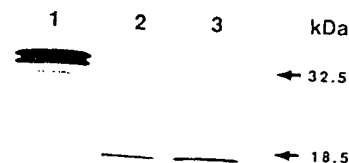


FIG. 4. Cleavage of pIL-1 $\beta$  by allelic variants of streptococcal cysteine protease. Rabbit reticulocyte lysate containing [ $^{35}$ S]methionine-labeled pIL-1 $\beta$  (lane 1) was incubated with SPE B purified from strain 279 (lane 2) or strain 289 (lane 3) as described in the legend to Fig. 2A.

Holm *et al.* (10) have clearly demonstrated that patients with fatal streptococcal infection have lower acute antibody levels to SPE B than do individuals with less severe infections.

Nitric oxide production by vascular wall cells via an IL-1 $\beta$ -mediated pathway may trigger a second, perhaps distinct, pathogenesis cascade for many of the signs and symptoms recorded in severe streptococcal infections. Clearly, additional studies are required to elucidate the virulence role, if any, of cleavage of pIL-1 $\beta$  to form mIL-1 $\beta$ .

Our data provide a further dimension to an important emerging theme in microbial pathogenesis that bacteria and viruses synthesize products that directly interact with cytokines and thereby potentially modulate the host immune system (33). For example, vaccinia and cowpox viruses encode a secreted 33-kDa IL-1 $\beta$  binding protein (34, 35) that exhibits 30% amino acid identity to the type II IL-1 receptor, a 63-kDa protein generally found on B cells and monocytes. The protein, designated B15R, can block IL-1-induced proliferation of murine B lymphocytes and thymocytes. A recombinant vaccinia virus containing the inactivated structural gene for B15R was less virulent in a mouse model of vaccinia virus disease. Cowpox virus synthesizes a 38-kDa protein termed cytokine response modifier A (CRM A) protein that is a member of the serpin family. Ray and colleagues (36) have shown that CRM A inhibits the endogenous human IL-1 $\beta$  converting enzyme that cleaves pIL-1 $\beta$  to produce mIL-1 $\beta$ . Several other viral proteins, including a vaccinia virus molecule that binds to the complement component C4b and blocks the classical complement cascade pathway, and a soluble receptor for tumor necrosis factor made by Leporipoxviruses, are virulence factors with ability to modulate the host immune system. Myxoma virus was recently shown to encode a homolog of the interferon  $\gamma$  receptor (37). Finally, *Pseudomonas aeruginosa* alkaline protease and elastase both can inactivate human interferon  $\gamma$  and tumor necrosis factor  $\alpha$ , but neither enzyme inactivates IL-1 $\alpha$  or IL-1 $\beta$  (38).

Our results also contribute to a growing literature suggesting that extracellular cysteine proteases made by human microbial pathogens are important in host-parasite interactions. Recently, considerable research has focused on characterization of eukaryotic parasite cysteine proteases that are widely believed to be virulence factors (39). For example, *Trypanosoma cruzi*, the agent of Chagas disease, expresses a cysteine protease termed cruzipain that is developmentally regulated (40), is a major antigen (41, 42), and elicits T-cell proliferatory responses in most chronically infected patients (43). Similarly, virulent strains of *Entamoeba histolytica* synthesize an abundant cysteine protease that cleaves extracellular matrix proteins and potentially induces cytopathic effects in the host (44, 45). Interestingly, the cysteine protease made by *T. cruzi*, like the *S. pyogenes* enzyme, is synthesized as an inactive precursor and can undergo autolytic degradation to produce a truncated active form (46, 47). Taken together, the data suggest similarities between the streptococcal cysteine protease and those made by other important microbial pathogens. Additional investigations are necessary to assess the extent of the shared characteristics.

The purified SPE B variants we tested for pIL-1 $\beta$  cleavage ability represent alleles that together are expressed by natural *S. pyogenes* clones frequently responsible for TSS, scarlet fever, acute rheumatic fever, and pharyngitis (V.K. and J.M.M., unpublished data). It is therefore likely that most and perhaps all *S. pyogenes* isolates can generate biologically active IL-1 $\beta$  from pIL-1 $\beta$  under appropriate circumstances. Our discovery suggests that cleavage of other host cytokines by SPE B might also have an important role in streptococcal pathogenesis and suggests potentially useful approaches for therapeutic intervention in the molecular pathogenesis pathway.

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